Bacterial lipoprotein tolerance attenuates cardiac dysfunction in septic mice

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Abstract
In order to examine the effect of BLP tolerance on cardiac dysfunction in CLP-induced septic mice, cardiac function was measured via echocardiography at various time points following induction of sepsis by CLP in BLP-tolerant and control C57BL/6 mice. Levels of tumour necrosis factor α (TNF-α), interleukin 10 (IL-10) and Toll-like receptor 2 (TLR2)-myeloid differentiation factor 88 (MyD88)-dependent signaling molecules in the myocardium were also examined. Cardiac function was significantly decreased 6-12 h following CLP-induced sepsis compared with that of sham controls, with cardiac output decreasing from 0.50 ±0.06 ml/s to 0.16 ±0.04 ml/s at 6 h and 0.57 ±0.06 ml/s to 0.13 ±0.02 ml/s at 12 h, whereas BLP tolerance attenuated CLP-induced cardiac dysfunction at 12 h following CLP with cardiac output increasing from 0.13 ±0.02 ml/s to 0.26 ±0.05 ml/s at 12 h. TNF-α, IL-10 and TLR2-MyD88-dependent signaling molecules in the myocardium were not significantly different among BLP-tolerant or non-tolerant control groups. These findings suggest that low dose BLP pretreatment can attenuate cardiac dysfunction, increasing the survival rate of septic mice. This effect did not involve TNF-α, IL-10 or TLR2-MyD88-dependent cytokines. Bacterial lipoprotein pretreatment attenuated cardiac dysfunction in CLP sepsis. TNF-α, IL-10 and TLR2-MyD88-dependent signaling molecules did not contribute to cardiac dysfunction observed in CLP-induced sepsis.

Key words: cardiac dysfunction, bacterial lipoprotein, Toll-like receptor 2, sepsis.

Introduction
Sepsis results from systemic inflammation and can lead to lethal organ damage. Despite recent advances in anti-biotic therapy and intensive care, overall mortality from severe sepsis has exceeded 30% of the total mortality in the United States [1-3]. Current research has shown that after severe injury or infectious challenges, some patients respond by the activation of proinflammatory signaling pathways and overexpression of inflammatory mediators, resulting in a systemic inflammatory response. This culminates in severe shock, multi-organ failure, and death [4]. Cardiovascular dysfunction is a major consequence of septic shock and contributes to the high morbidity and mortality rates observed in septic patients [5, 6]. Sepsis-induced cardiovascular dysfunction is characterized by decreased contractility, impaired ventricular response to fluid therapy, and ventricular dilatation [7]. Recent studies have shown that circulating cytokines (TNF-α, IL-1β), lysozyme c, or endothelin-1 have direct inhibitory effects on myocyte contractility. Intracellular alterations in calcium flux within the cardiomyocyte can also influence myocyte contractility during sepsis [8]. Despite extensive basic and clinical research, the pathophysiology of myocardial dysfunction associated with septic shock is still poorly understood.

Bacterial lipoprotein (BLP), characterized by a unique NH2-terminal lipo-amino acid, N-acyl-S-diacylglyceryl cysteine, is the most abundant protein in the outer membrane of both gram-positive and gram-negative bacteria. BLP is known to activate monocytes and/or macrophages to produce inflammatory cytokines and to induce apoptosis in experimental animals [9, 10]. Pre-exposure to BLP induces

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BLP tolerance, which can protect mice against BLP, lipopolysaccharide- (LPS), live bacterial-, and polymicrobial sepsis-induced lethality [11].

Families of the Toll-like receptors (TLRs) are involved in BLP tolerance. TLRs have recently emerged as key components of the innate immune system that detect microbial infection and trigger antimicrobial host defense responses [12]. TLR2 regulates the cellular expression of proinflammatory mediators following BLP stimulation [13]. BLP activates the TLR2 signaling cascade through the TLR2 Toll/IL-1 receptor (TIR) domain, using a conserved signal transduction pathway requiring myeloid differentiation factor 88 (MyD88), IL-1-receptor-associated kinases (IRAKs), tumor necrosis factor receptor-associated factor 6 (TRAF6), IκB kinase kinase (IKK), and nuclear factor-κB (NF-κB) to induce tumor necrosis factor-α (TNF-α) or interleukin (IL)-10 production. Recent studies have shown that modulation of this signaling pathway via TLR2 is involved in the development of BLP tolerance [14, 15]. Pretreatment of human THP-1 monocyte cells with BLP induced tolerance to a second BLP challenge with diminished TNF-α and IL-6 production. BLP tolerance is associated with reductions in TLR2 and IRAK-1 expression, MyD88-IRAK complex formation and IκB-α phosphorylation. However, the role of TLR2 signaling in the tissue response to septic shock in BLP-tolerant mice has not been determined.

In the present study, we investigated the protective effect of BLP tolerance in septic shock and examined whether TNF-α and IL-10 production correlates with myocardial dysfunction seen in septic shock. Furthermore, we assessed the effects of a TLR2-mediated signaling pathway in the cardiac parenchymal cells of BLP-tolerant mice.

Material and methods

Reagents and antibodies

BLP, a synthetic BLP (Pam3Cys-Ser-(Lys)4•3HCl) was purchased from Alexis Biochemicals (San Diego, CA). Polyclonal antibodies (pAb) for TLR2, IRAK-1, IKKα, IκB-α and NF-κB p65 were obtained from Cell Signaling Technology (Beverly, MA). Anti-MyD88 pAb was purchased from eBioscience (San Diego, CA). Rabbit pAb against TRAF6 (Mid) was obtained from Zymed Laboratories (South San Francisco, CA). mouse pAb for α-tubulin was purchased from Sigma-Aldrich (St. Louis, MO), and ELISA tests for mouse TNF-α and IL-10 were performed using commercially available kits (BioSource International, Camarillo, CA). The TUNEL assay kit was purchased from Promega (Madison, WI).

Animals and CLP polymicrobial sepsis model

Pyrogen-free male C57BL/6 mice (6–8 wk old; 19–26 g) were obtained from Shanghai SLAC Laboratory Animal CO. Ltd (PRC). The mice were quarantined and maintained on a standard pellet diet for 1 wk prior to any experiments. All animal procedures were performed in accordance with the standards of the Animal Use and Care Committee of Nanjing Medical University (PRC). Tolerance in mice was induced by injection of 10 mg/kg BLP (i.p., BLP tolerance) [11], or an equal volume (200 μl) of PBS (no tolerance) 24 h prior to septic challenges. Following BLP tolerance induction, non-tolerant and BLP-tolerant mice were subjected to polymicrobial sepsis, which was induced by cecal ligation and puncture (CLP). Briefly, the mice were anesthetized with chloral hydrate, a midline incision was made on the anterior abdomen, and the cecum was exposed and ligated with a 3–0 silk suture. Two punctures through the cecum were made with an 18-gauge needle, and feces were extruded from the holes. The cecum was then returned to the peritoneal cavity and the abdominal incision was closed. Sham-operated mice served as surgical controls; these animals were anesthetized, a midline incision was made, the cecum was exposed for one minute, and the abdominal incision was then closed. After sepsis induction, a single dose of resuscitative fluid (lactated Ringer solution, 25 ml/kg body weight) was immediately administered at room temperature by subcutaneous injection. There were four groups: the normal control (N), sham control (S), BLP tolerance + CLP (B + C) and CLP (C).

Experimental protocols

Mice were subjected to CLP at 0 h, 2 h, 6 h and 12 h following CLP; cardiac function measurements were performed as described previously [16, 17]. To examine the effects of BLP tolerance TLR2-mediated signaling and cardiac myocyte apoptosis, hearts were harvested at 0, 2, 6 and 12 h, respectively. Hearts were washed free of blood using iced-cold phosphate buffered saline (PBS) and a single tissue section (5 mm) was taken from each heart at the same anatomical location. The immersion was fixed in 4% buffered paraformaldehyde, and embedded in paraffin for tissue section preparation. The remaining heart tissue sections were immediately frozen in liquid nitrogen and stored at −70°C.

Cardiac function assessment

Echocardiography was performed using a 13 MHz linear array ultrasound probe (Vivid 7 Dimension, GE Medical, USA) in sedated mice (chloral hydrate, 200 mg/kg, i.p.). Parasternal short- and long-axis views were obtained at the midventricular level, at a frame rate of 483 frames per second and a depth of 1 cm. LV dimensions were measured from the M-mode tracings. Fractional shortening (FS), left ventricle ejection fraction (EF) and cardiac output (CO) were calculated with the use of a customized version of the EchoPac Software (GE Medical). Values obtained in three consecutive cardiac cycles were averaged. CO, EF, and hear rate (HR) were chosen as indices of cardiac function.
Western blot analysis

Sample preparation

Nuclear and cytoplasmic proteins were isolated from the whole heart tissue homogenates using a method described previously [18]. Briefly, myocardial samples were homogenized in 0.7 ml of ice-cold buffer A (5 mM Tris base HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10 mM EGTA pH 8.0, 1 mM dithiothreitol [DTT], protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany], and phosphatase inhibitors: 50 mM NaF, 30 mM β-glycerophosphate, 1 mM Na3VO4, and 20 mM ρ-nitrophenol-Na). Homogenates were centrifuged for 20 min at 10,000 rpm at 4°C. Supernatants containing cytoplasmic proteins were collected and stored at −70°C. The pellets were suspended in ice-cold buffer B (5 mM Tris•HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10 mM EGTA pH 8.0, 1 mM DTT, protease inhibitors, phosphatase inhibitors, 10% Nonidet P-40, 10% Brij-35, 10% deoxycholic acid sodium salt), incubated on ice for 60 min, mixed frequently, and centrifuged for 15 min at 7900 g maintained at 4°C. Supernatants were collected following centrifugation as nuclear extracts and stored at −70°C. The concentration of the total protein in each sample was determined using the Pierce protein assay reagent (Pierce Chemical, Rockford, IL).

Western blotting

Cytoplasmic and nuclear proteins were denatured at 100°C for 5 min in the loading buffer (60 mM Tris, 2.5% sodium dodecyl sulfate [SDS], 10% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue). Aliquots containing an equal amount of the total protein from each sample were separated in SDS-polyacrylamide gels and transferred onto immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h at room temperature with 5% nonfat milk and probed overnight (maintained at 4°C) with appropriate primary antibodies (pAb) at conditions recommended by the manufacturers (anti-TLR 2, anti-MyD88, anti-IRAK1, anti-TRAF6, anti-IKKα, anti-IκB-α and anti-NF-κB (p65)), respectively. Blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h, developed with SuperSignal chemiluminescent substrate (Pierce Chemical, Rockford, IL) and exposed to X-Omat BT films (Kodak). Quantification of the developed target bands was determined by densitometric analysis.

Myocardial cytokine measurements

TNF-α and IL-10 levels in the myocardial cytoplasmic proteins were determined using commercially available ELISA kits according to the manufacturer’s instructions.

In situ apoptosis assay

In situ cardiac myocyte apoptosis was examined using the TUNEL assay. This assay was performed according to the manufacturer’s instructions. Briefly, tissue sections were deparaffinized, rehydrated, fixed and permeabilized, and then incubated with FITC-labelled TdT incubation buffer at 37°C for 1 h. Anti-α-actinin immunohistochemistry was then performed. Sections were blocked, incubated with primary antibody against α-actinin for 1 h followed by incubation with Cy3-conjugated anti-IgG for 1 h. Hoechst33342 reagent was then used to counter stain the nuclei. The fluorescence images were observed and captured using a confocal microscope (2-Photon, LSM 510, Zeiss). Five slide fields were randomly examined using a defined rectangular field area with magnification ×200. One hundred cells were counted in each field, and apoptotic cardiac myocytes were presented as the percentage of total cells counted.

Statistical analysis

Results are expressed as mean ± SEM. Analysis of variance (ANOVA) was used to assess for differences between groups at each time-point, followed by a Scheffe multiple comparison for post hoc analysis. Differences between groups of relative intensity for western blot analysis were performed with repeated measures by using the random-effects generalized estimated equation (GEE). p < 0.05 was specified as statistically significant.

Results

BLP tolerance prevented cardiac dysfunction in septic mice

In vivo cardiac function was measured at 0 h, 2 h, 6 h and 12 h after the mice were subjected to CLP via echocardiography. As shown in Figs. 1 and 2, HR, FS and EF were increased between 0 h and 6 h, and then declined to the baseline at 12 h in shams. CO was decreased between 0 h and 2 h, and returned to the baseline at 12 h in the CLP group. HR and FS were increased between 0 h and 2 h, and decreased after 2 h. EF was increased at 2 h and decreased after 6 h. CO was continuously decreased at 12 h compared to the baseline. HR, FS, EF and CO in the BLP tolerance + CLP group were similar to the CLP group between 0 h and 6 h (p < 0.05). They were then increased after 6 h, which was significantly higher than in the CLP group at 12 h (p < 0.05, respectively). Interestingly, BLP tolerance prevented sepsis-induced cardiac dysfunction during the middle stage of sepsis.

Myocardial cytokine production following sepsis

Changes in TNF-α and IL-10 levels in the myocardial cytoplasmic proteins are shown in Fig. 3 and 4. TNF-α levels in the myocardium at 0 h, 2 h, 6 h and 12 h after CLP were not different from those of the control or sham groups.
2h after CLP, IL-10 levels in the myocardium were significantly increased in the BLP tolerance + CLP group as compared with levels in the sham group (p < 0.05) and in the CLP mice (p < 0.05), respectively. However, IL-10 levels were not different at any other time points among the four groups (p > 0.05).

**Protein expression of TLR2, MyD88, IRAK-1, TRAF6, IKK-α, IκB-α and NF-κB (p65) in the myocardium of CLP-induced septic mice**

*In vitro* BLP tolerance developed through the down-regulation of TLR2 expression [12]. We examined myocardial protein levels of TLR2, MyD88, IRAK-1, TRAF6, IKK-α, IκB-α and NF-κB (p65) by Western blot analysis in BLP-tolerant and non-tolerant septic mice. Figs. 5 and 6 show that BLP pretreatment did not alter TLR2, Myd88, IRAK-1, IKK-α or NF-κB myocardial expression among the four groups (p > 0.05, respectively).

In Fig. 5, the levels of TRAF6 in the myocardium of BLP-tolerant mice at time 0 h were significantly higher than in the control group, and not markedly different from that in the sham group and non-tolerant CLP mice at times 2 h, 6 h and 12 h after CLP.

As shown in Fig. 6, IκB-α level in the myocardium of BLP-tolerant septic mice at the time 12 h following CLP were significantly increased compared with the levels of the sham and CLP groups. However, there was no significant difference at other time points among the four groups.

**Apoptosis in cardiomyocytes is delayed by BLP tolerance**

Apoptosis in mouse cardiomyocytes was measured at 0 h, 2 h, 6 h and 12 h after the mice were subjected to CLP *via* TUNEL assay. As shown in Fig. 7, there were more apoptotic cells in BLP tolerance than in the normal or the control groups (p < 0.05), however after CLP challenge,
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Fig. 2. BLP tolerance prevented cardiac dysfunction in CLP-induced septic mice. Echocardiographic parameters were examined at time 0 h, 2 h, 6 h and 12 h after CLP. There were five mice in each group.
apoptosis in mouse cardiomyocytes increased rapidly, with no significant changes in the BLP-tolerance mice at the corresponding time points. These results imply that BLP tolerance may induce apoptosis in cardiomyocytes, but may delay apoptosis triggered by CLP.

**Discussion**

BLP tolerance attenuated LV cardiac dysfunction in mice with CLP-induced sepsis. BLP attenuation of cardiac dysfunction had no significant correlation with TNF-α, IL-10 or TLR2-mediated signaling in the myocardium, however BLP tolerance can delay CLP-triggered apoptosis in the mouse myocardium.

Septic shock is a serious problem that is routinely encountered in the intensive care units of hospitals. Despite effective antibiotics, the lethality of septic shock remains high [19]. This problem is likely to increase as more aggressive treatment strategies are used in immunosuppressed patients. Although knowledge of the pathophysiology of septic shock continuously increases, its complexity is far from being understood. It is, however, accepted that once invading bacteria have triggered septic shock, therapeutic interventions may often be too late. Strategies thus need to be developed to prevent the pathophysiological response of the infected host [20].

The CLP septic shock model used in the present study is a hypodynamic sepsis model that is characterized by reduced CO and EF. In this study, CLP induced a progressive decrease in FS, CO and HR from 2 h to 12 h following surgery. At the same time, we observed a slight increase in EF between 2 and 6 h after CLP, and an abrupt decrease between 6 and 12 h.

**In vivo**, tolerance induced by BLP can protect mice against not only lethality, but also LPS-, live bacterial-, and polymicrobial sepsis-induced lethality [11]. **In vitro**, THP-1 cells are pretreated with a low dose of BLP for 24 h and then subjected to a second stimulation with the same or higher doses of BLP. This causes TNF-α production to be reduced significantly [14, 15]. Experimental and clinical studies have shown that harmful tissue events, including infections, are perceived by macrophages and monocytes, which in turn secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and anti-inflammatory cytokines such as IL-4, IL-10 [11, 21-23]. Appropriate levels of these cytokines are essential for cell-mediated microbicidal activity as excessive production can lead to an uncontrolled inflammatory response, multiple organ failure, and ultimately death [11]. Experimental studies support a critical role for enhanced production of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and anti-inflammatory cytokines such as IL-4, IL-10 [11, 21-23]. Appropriate levels of these cytokines are essential for cell-mediated microbial activity as excessive production can lead to an uncontrolled inflammatory response, multiple organ failure, and ultimately death [11]. Experimental studies support a critical role for enhanced production of inflammatory cytokines in the development of sepsis-induced myocardial dysfunction [21, 24-26]. Meanwhile, cardiac tissues synthesize TNF-α, IL-1β, and IL-10 [5, 27-31] and increase the local production of cytokines, which has been implicated in myocardial dysfunction observed during several pathological conditions [5, 30-32]. Therefore, we postulated that BLP administration could also improve myocardial function in the sepsis mice. To evaluate this hypothesis,
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Cardiac function and myocardial TNF-α and IL-10 levels in cytoplasmic proteins were measured from septic mice with or without BLP tolerance. We showed that cardiac function was significantly depressed in BLP-tolerant septic mice at 2 h and 6 h following CLP, and returned to the baseline at 12 h. BLP-tolerant septic mice cardiac function was significantly improved compared to that of non-tolerant septic mice at 12 h. Therefore, low dose BLP pretreatment resulted in enhanced cardiac function and increased survival in septic mice. However, myocardial IL-10 levels were significantly increased in the BLP tolerant septic mice as compared with those of the sham group and non-tolerant septic mice.

Fig. 5. Influence of BLP tolerance on the expression of the TLR2, MyD88, IRAK-1, TRAF6 in the myocardium of BLP-tolerant and non-tolerant septic mice. pTRAF6 protein expression by western blot analysis. There were three mice in each time point.

*p < 0.05 BLP+CLP versus the normal control (N). Results shown represent one experiment from a total of three independent experiments.
Fig. 6. Influence of BLP tolerance on the expression of the IKK-α, IκB-α and NF-κB in the myocardium of BLP-tolerant and non-tolerant septic mice. Cytoplasmic proteins were extracted for the examination of IKK-α, IκB-α and NF-κB protein expression by western blot analysis. There were three mice in each time point

TLRs are pattern recognition receptors (PPRs), which recognize distinct molecular patterns associated with microbial pathogens [12, 13]. For example, TLR2 is involved in gram-positive BLP stimulation, and TLR4 is required for gram-negative LPS signaling. Upon engagement by different microbial ligands, TLRs are activated and initiate a cascade of intracellular signaling that eventually result in inflammatory cytokine production. TLR2 recruits MyD88-dependent pathways [13, 33]. In vitro, Western blot analy-
Fig. 7. Effects of BLP tolerance on cardiomyocyte apoptosis. CLP-induced apoptosis was determined by the TUNEL assay. Hearts were harvested at 0, 2, 6, 12 h, and fixed and cut into 4 µm serial paraffin sections, respectively. The fluorescence images were captured from the left ventricular free wall. α-actinin was used to confirm that the cells were myocytes. Representative images were from three independent experiments. → indicates positive nuclei of TUNEL staining. Magnification 1000× Continued on next page
Recent studies have shown that TLR2 is expressed in the myocardium [29, 34-36]. In the present study, we observed a relationship between cardiac function and myocardial TLR2 signaling in septic mice. Although cardiac function was significantly depressed in CLP mice, there were no significant differences between BLP-tolerant and non-tolerant mice before or after a subsequent polymicrobial challenge. Therefore, despite the critical function of the TLR2-MyD88-dependent downstream pathway in trans-

sis demonstrated that there was a slight reduction in TLR2 expression in BLP-tolerant THP-1 cells and there were no significant alterations in protein levels of MyD88, IRAK-4, or TRAF6 in naive and BLP-tolerant cells, before or after BLP or LPS stimulation. Similarly, mRNA levels of TLR2 and TLR2 downstream signaling intermediates were not regulated significantly by BLP or LPS stimulation in naive or BLP-tolerant cells by quantitative real-time RT-PCR analysis [13].

Fig. 7. Effects of BLP tolerance on cardiomyocyte apoptosis. CLP-induced apoptosis was determined by the TUNEL assay. Hearts were harvested at 0, 2, 6, 12 h, and fixed and cut into 4 μm serial paraffin sections, respectively. The fluorescence images were captured from the left ventricular free wall. α-actinin was used to confirm that the cells were myocytes. Representative images were from three independent experiments. → indicates positive nuclei of TUNEL staining. Magnification 1000×

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